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Rapid Detection, Identification, and Enumeration of *Escherichia coli* Cells in Municipal Water by Chemiluminescent In Situ Hybridization

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A new chemiluminescent in situ hybridization (CISH) method provides simultaneous detection, identification, and enumeration of culturable *Escherichia coli* cells in 100 ml of municipal water within one working day. Following filtration and 5 h of growth on tryptic soy agar at 35°C, individual microcolonies of *E. coli* were detected directly on a 47-mm-diameter membrane filter using soybean peroxidase-labeled peptide nucleic acid (PNA) probes targeting a species-specific sequence in *E. coli* 16S rRNA. Within each microcolony, hybridized, peroxidase-labeled PNA probe and chemiluminescent substrate generated light which was subsequently captured on film. Thus, each spot of light represented one microcolony of *E. coli*. Following probe selection based on 16S ribosomal DNA (rDNA) sequence alignments and sample matrix interference, the sensitivity and specificity of the probe Eco16S07C were determined by dot hybridization to RNA of eight bacterial species. Only the rRNA of *E. coli* and *Pseudomonas aeruginosa* were detected by Eco16S07C with the latter mismatch hybridization being eliminated by a PNA blocker probe targeting *P. aeruginosa* 16S rRNA. The sensitivity and specificity for the detection of *E. coli* by PNA CISH were then determined using 8 *E. coli* strains and 17 other bacterial species, including closely related species. No bacterial strains other than *E. coli* and *Shigella* spp. were detected, which is in accordance with 16S rDNA sequence information. Furthermore, the enumeration of microcolonies of *E. coli* represented by spots of light correlated 92 to 95% with visible colonies following overnight incubation. PNA CISH employs traditional membrane filtration and culturing techniques while providing the added sensitivity and specificity of PNA probes in order to yield faster and more definitive results.

Drinking water is a critical part of the human diet, and contamination of municipal water with pathogenic microorganisms constitutes a serious threat to the public health (10, 16). Numerous examples of disease outbreaks due to contamination of municipal water with microorganisms have been reported worldwide (14), including recent outbreaks of *Escherichia coli* O157 in Albany, N.Y., and Walkerton, Canada (4, 13), and regulations for microbial limits in drinking water have been issued (1).

The coliforms comprise a group of gram-negative bacteria producing acid and gas from lactose within 48 h of incubation at 35°C. Their presence is a widely accepted indicator for fecal contamination. The concept of coliforms has been further refined by differentiating between environmental coliforms and fecal coliforms exhibiting thermotolerance, such as *E. coli*. The development of *E. coli*-specific tests based on either detection of β-D-glucoronidase activity using media containing 4-methylumbelliferyl-β-D-glucuronide (MUG) (7) or molecular methods (2, 22) now allows fecal contamination to be monitored by specific detection of *E. coli*. In this way, no further identification is required and positive reactions due to coliforms not associated with fecal contamination are eliminated.

Current standard methods for detection of coliforms and *E. coli* rely on growth to visible colonies and therefore take a minimum of 18 h. The importance of rapid detection of *E. coli* with regard to quality and safety of municipal water is obvious. Rapid detection methods for *E. coli* have previously been described and include biochemical methods such as ATP-based bioluminescence (S. D. Upperman et al., Abstr. 99th Gen. Meet. Am. Soc. Microbiol., abstr. O-14, 1999) and methods such as PCR (2) and peptide nucleic acid (PNA) probe hybridization followed by signal amplification (18). However, none of these rapid methods provides simultaneous detection, identification, and enumeration.

PNA probes are DNA mimics with a polyamide backbone to which the individual nucleobases are attached (15). This enables PNA probes to hybridize to complementary nucleic acid targets obeying Watson-Crick base pairing rules with high specificity and rapid binding kinetics (8). These properties are ascribed to the uncharged backbone of PNA probes and have opened possibilities for new diagnostic assays within different areas of microbiology. In particular, PNA probes targeting species-specific rRNA sequences for definitive identification of microorganisms by fluorescence in situ hybridization have recently been published (19, 21, 23). These applications all utilize rRNA as target due to its high cellular abundance, universal distribution, and use as a phylogenetic marker (6, 11).

In this study, PNA chemiluminescent in situ hybridization (CISH), a newly described CISH method using PNA probes for the rapid and simultaneous detection, identification, and enumeration of microorganisms (17, 19), was applied to the analysis of *E. coli* in municipal water within one working day.

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TABLE 1. Results of *E. coli* PNA CISH and culture following 5-h and overnight incubation, respectively

Organism	Strain	PNA CISH	Culture[a]
Escherichia coli	ATCC 8739[b]	+	+
Escherichia coli	GMF/MSU[c]	+	+
Escherichia coli	WWTx[d]	+	+
Escherichia coli	MWTX[d]	+	+
Escherichia coli	ATCC 25922	+	+
Escherichia coli	HF174	+[e]	+
Escherichia coli	MSC1 S[d]	+	+
Escherichia coli	MSC2 L[d]	+	+
Shigella sonnei	ATCC 29930	+	+
Shigella flexneri	ATCC 29903	+	+
Hafnia alvae	ATCC 13337	−	+
Pseudomonas putida	ATCC 12633	−	+
Serratia marcescens	ATCC 13880	−	+
Pseudomonas aeruginosa	ATCC 27853	−	+
Bacillus subtilis	ATCC 6633	−	+
Staphylococcus epidermidis	ATCC 14490	−	+
Staphylococcus aureus	ATCC 6538	−	+
Listeria innocua	ATCC 33090	−	+
Salmonella enterica serovar Choleraesuis	ATCC 29946	−	+
Acinetobacter calcoaceticus	ATCC 23065	−	+
Citrobacter freundii	ATCC 8090	−	+
Enterobacter aerogenes	ATCC 49701	−	+
Klebsiella pneumoniae	GMF/MSU[c]	−	+
Proteus mirabilis	ATCC 12453	−	+
Micrococcus luteus	ATCC 9341	−	+

[a] +, positive reaction; −, negative reaction.
[b] ATCC, American Type Culture Collection, Manassas, Va.
[c] Strains kindly provided by Gordon McFeters, Montana State University.
[d] Environmental isolates from culture collection of Millipore Corporation.
[e] Weak result.

Municipal water samples were filtered to isolate and separate individual microorganisms onto membrane filters, which were then placed on culture media for 5 h prior to testing. Microcolonies of *E. coli* were detected on the membrane filter by in situ hybridization with peroxidase-labeled PNA probes targeting *E. coli* 16S rRNA. Unhybridized probe was removed by washing, and hybridized probe was visualized by a chemiluminescent reaction. Each microcolony of *E. coli* was observed as a spot of light, providing simultaneous detection, identification, and enumeration.

MATERIALS AND METHODS

Bacterial strains and municipal water samples. Eight *E. coli* strains and 19 other bacterial strains representing environmentally and clinically relevant bacterial species were obtained from various sources (Table 1). Unless otherwise noted, *E. coli* ATCC 8739 was the representative strain for all experiments. The strains were propagated in either Luria-Bertani (LB) broth base (Sigma Chemical Co., St. Louis, Mo.) or tryptic soy broth (Sigma or Difco Laboratories, Detroit, Mich.) at 30 to 35°C. Municipal water samples were obtained from four different tap water faucets in eastern Massachusetts. Filter sterilizations of municipal water samples were performed with SteriCups containing Durapore filters of 0.22 μm in pore size (Millipore Corporation, Bedford, Mass.).

Sample preparation. Prior to filtration, strains were diluted in filter-sterilized phosphate-buffered saline or 0.15 M NaCl. For correlation studies, municipal water samples were treated to include 0.01% (wt/vol) sodium thiosulfate (Sigma) in order to neutralize free chlorine. All samples were filtered through 47-mm-diameter polyvinylidene difluoride membrane filters with pore sizes of 0.45 μm (Millipore Corporation). Membrane filters were aseptically transferred using forceps to petri dishes containing tryptic soy agar (TSA) (Difco Laboratories), LB agar (Difco Laboratories), or R2A (Difco Laboratories) and incubated for 5 h at 4 or 35°C prior to PNA CISH analysis as described below. For all experiments, samples were incubated on TSA at 35°C for 5 h unless otherwise noted. Visible colony counts were obtained following overnight incubation at 35°C.

Selection of probe sequence. Sequence processing was performed using computer software from DNASTAR (Madison, Wis.). Alignments of closely related 16S ribosomal DNA (rDNA) sequences were performed using Megalign (version 4.03) software. From such alignments, species-specific target sequences of *E. coli* were identified and subsequently checked for any significant sequence similarity against the whole GenBank database using GeneMan (version 3.30) software and Advanced BLAST searches (version 2.0 [www.ncbi.nlm.nih.govlast]). Probe sequences were then checked for any significant level of internal secondary structure using PrimerSelect (version 4.03) software.

Synthesis of soybean peroxidase (SBP)-labeled PNA probes. Unlabeled PNA oligomers were synthesized using an Expedite 8909 nucleic acid synthesis system with PNA option and reagents (PE Biosystems, Foster City, Calif.). The aqueous solubility of the PNA was enhanced by flanking the nucleobase sequence with two solubility enhancers (12) at the C terminus, while the N terminus of the PNA was extended using two 8-amino-3,6-dioxaoctanoic acid spacers (PE Biosystems) and one 4-aminobenzoic acid linker (PE Biosystems). The PNA was then purified by reverse-phase C_{18} chromatography with a 300- by 7.8-mm Delta Pak column of 15 μm in particle size and 300 Å in pore size (Waters Corporation, Milford, Mass.). The 4-aminobenzoic acid linker of the PNA probe was conjugated to SBP (Wiley Organics, Columbus, Ohio) using a recently published method (J. Coull and R. Fitzpatrick, 1999, PCT patent application WO1999US0002908), after which the PNA-SBP conjugate was purified from nonconjugated enzyme and excess PNA by size exclusion chromatography (20). The UV absorbances at 260 and 405 nm of the conjugate were measured in order to determine the yield. The PNA-SBP conjugate was lyophilized, and the resulting material was reconstituted in peroxidase stabilization buffer (Dako Diagnostics, Mississauga, Canada) to provide a solution of PNA-SBP conjugate with a concentration of 10 μM.

Dot hybridization to bacterial RNA. RNA was purified from exponentially growing bacterial cells using the RNA/DNA Maxi kit (Qiagen, Chatsworth, Calif.). Ten nanograms of purified RNA was spotted onto Gibco BRL Biodyne A membranes (Life Technologies, Gaithersburg, Md.) and cross-linked using 33 mJ of UV irradiation in a Stratolinker (Stratagene, La Jolla, Calif.). Hybridization with SBP-labeled PNA probes was performed using the PNA Micro Dx dot blot reagent kit (Boston Probes, Bedford, Mass.). Briefly, membranes were prehybridized with hybridization buffer for 15 min at 50°C and then hybridized with hybridization buffer containing 0.6 nM SBP-labeled PNA probe with or without 60 nM PNA blocker probe for 30 min at 50°C. Unhybridized SBP-labeled PNA probe was removed by washing the membranes four times for 7 min each at 50°C with prewarmed wash solution. Hybridized probe was visualized by placing the membranes for 2 min in SuperSignal chemiluminescent substrate (Pierce Chemical Company, Rockford, Ill.) and exposing them to Super RX medical X-ray film (Fuji, Tokyo, Japan) for 15 min.

CISH using PNA probes (PNA CISH). Prior to hybridization, microcolonies were fixed to the membrane filter by placing the membrane filter on a 47-mm absorbent pad (Millipore) soaked with 1.5 ml of fixation solution (0.35% [vol/vol] glutaraldehyde [Sigma], 5 mM sodium azide [Sigma], 0.01% [wt/vol] urea H_2O_2 [Sigma], 90% [vol/vol] denatured ethanol [VWR Scientific Products Corporation, Boston, Mass.]) for 5 min at room temperature. Hybridization was performed for 30 min at 50°C in a covered Petrislide (Millipore) using 1.5 ml of 3 nM SBP-labeled PNA probe with or without 300 nM PNA blocker probe in hybridization solution (25 mM Tris [J. T. Baker, Phillipsburg, N.J.] [pH 9.5], 50% [vol/vol] formamide [Sigma], 0.7% [vol/vol] Tween 20 [Sigma], 2% [wt/vol] polyvinylpyrrolidone [average molecular weight of 10,000] [Sigma], 1% [wt/vol] yeast extract [Difco Laboratories], 1% [wt/vol] casein [Sigma], 0.1 M NaCl [J. T. Baker], 5 mM EDTA [Sigma]). Unhybridized probe was removed hy washing the membrane filter four times for 7 min each at 50°C in wash solution (10 mM CAPSO [Sigma] [pH 10.0] or 10 mM CAPSO [Sigma] [pH 10.5], 0.2% [vol/vol] Tween 20 [Sigma]. Washing was performed using a PNA wash rack in a wash container (Boston Probes). Hybridized probe was visualized by placing the membrane filter for 2 min in a mixture of 150 μl of Luminol enhancer and 150 μl of stable peroxidase, both part of the FemtoWest chemiluminescent substrate (Pierce Chemical Company). Membrane filters were subsequently placed in Fotolopes (Boston Probes), and light generated from the reaction was detected by a 15-min exposure to Super RX medical X-ray film (Fuji) developed by an M35A X-OMAT processor (Kodak, Rochester, N.Y.).

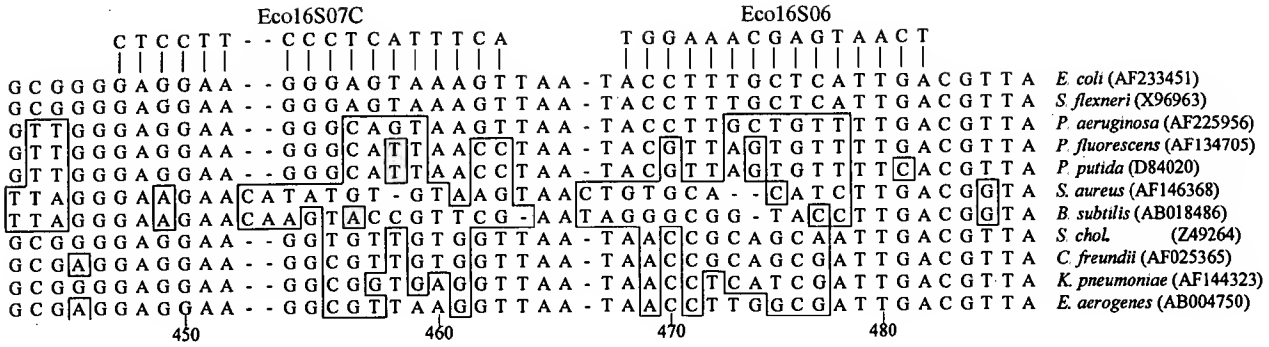

FIG. 1. Alignment of partial bacterial 16S rDNA sequences for indicated species. Accession numbers are included in parentheses. Antiparallel hybridizations of Eco16S06 and Eco16S07C to target 16S rDNA sequences are shown above the alignment. The target sequences of Eco16S06 and Eco16S07C correspond to *E. coli* 16S rDNA positions 468 to 482 and 447 to 463, respectively, according to the numbering of Brosius et al. (3). *S. chol*, *Salmonella enterica* serovar Choleraesuis.

RESULTS

PNA probe selection. Sequences of 16S rDNA from *E. coli* and 10 other bacterial species were aligned in order to identify species-specific target regions of *E. coli* 16S rDNA. Two different probes, Eco16S06 and Eco 16S07C, were designed. These probes targeted sequences that were identical to all published sequences of *E. coli* and differed by several bases from sequences from other bacterial species, except for *Shigella* species (Fig. 1). BLAST searches confirmed the specificity of the target sequences, with the exception that one of four published 16S rDNA sequences of *Hafnia alvea* (accession no. Z83203) and one of 12 published 16S rDNA sequences of *Serratia marcescens* (accession no. AF076038) contained this target sequence.

Sample matrix interference. Initially, the two SBP-labeled PNA probes were tested by the PNA CISH method for nonspecific binding to material in municipal water samples deposited on the membrane filter following filtration. It was found that Eco16S06-SBP gave rise to unacceptably high, nonspecific binding, especially when large sample volumes were filtered (Fig. 2). This nonspecific binding was observed as spots whose appearance was similar to true-positive spots from the reaction of the SBP-labeled PNA probe to microcolonies of *E. coli* (see Fig. 5) and was therefore likely to be mistaken for a true-positive reaction. In contrast, Eco16S07C-SBP showed no interference with sample matrix material (Fig. 2). These data were reproduced using different syntheses of SBP-labeled PNA probes and water samples from four different locations.

In an attempt to reduce the number of nonspecific background spots obtained with Eco16S06-SBP, it was found that adding 1% (wt/vol) yeast extract to the hybridization buffer significantly decreased the number of background spots without decreasing the probe-specific signal. From these experiments, only Eco16S07C-SBP was selected for further experiments, and 1% (wt/vol) yeast extract was included in the hybridization buffer.

Probe sensitivity and specificity. The theoretical specificity of Eco16S07C-SBP based on sequence analysis was evaluated by dot hybridization using purified RNA from eight bacterial species (Fig. 3). Results using the universal bacterial PNA probe (BacUni1-SBP) (17) served as the control for the presence of 16S rRNA for all bacteria. Although Eco 16S07C-SBP reacted strongly with *E. coli*, it also showed pronounced cross-hybridization to *P. aeruginosa*, but not to any of the other six species, including other *Pseudomonas* and *Pseudomonas*-like species. From the sequence alignment shown in Fig. 1, numerous mismatches were noted between the probe and the 16S rDNA sequence of *P. aeruginosa*. However, a closer examination revealed that the exclusion of a C (position 456) in the *P. aeruginosa* 16S rDNA sequence and a T (position 459) of Eco16S07C-SBP generated 16 bases of complete complementarity between Eco16S07C-SBP and the 16S rDNA sequence of *P. aeruginosa*.

In an attempt to eliminate this mismatch hybridization, an

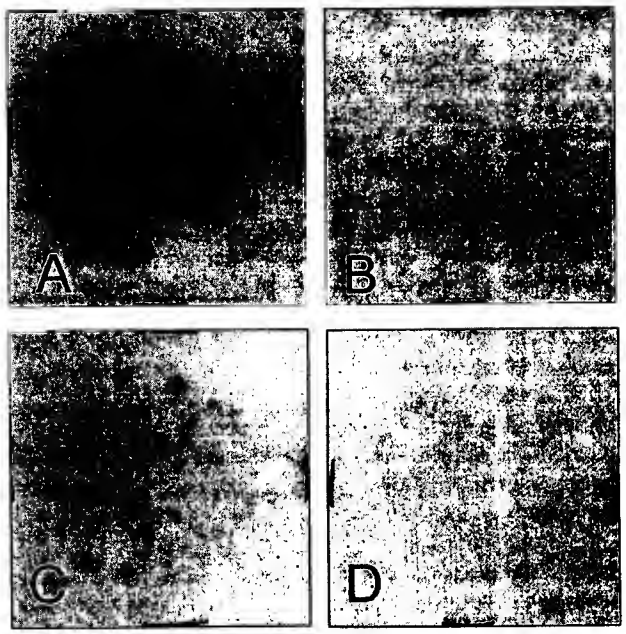

FIG. 2. Images of 500ml of municipal water as analyzed by PNA CISH using different PNA probes and hybridization buffer with and without 1% (wt/vol) yeast extract. (A) Eco16S06-SBP, no yeast extract; (B) Eco16S07C-SBP, no yeast extract; (C) Eco16S06-SBP, yeast extract; (D) Eco16S07C-SBP, yeast extract.

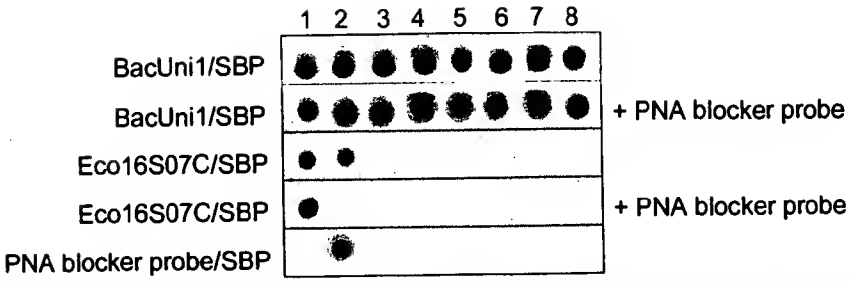

FIG. 3. Dot hybridizations of BacUni1-SBP, Eco16S07C-SBP, and PNA blocker probe-SBP with and without PNA blocker probe to purified RNA from *E. coli* ATCC 25922 (lane 1), *P. aeruginosa* ATCC 27853 (lane 2), *Pseudomonas fluorescens* ATCC 13525 (lane 3), *Pseudomonas putida* ATCC 12633 (lane 4), *Burkholderia cepacia* ATCC 25416 (lane 5), *S. aureus* ATCC 6538 (lane 6), *Bacillus subtilis* ATCC 6633 (lane 7), and *Salmonella enterica* serovar Choleraesuis ATCC 29946 (lane 8). The concentration of PNA blocker probe is 100-fold in excess of SBP-labeled probe. Each spot of purified RNA is 10 ng.

unlabeled PNA blocker probe (ACTTACTGCCCTTCC) complementary to positions 449 to 463 of the 16S rDNA sequence of *P. aeruginosa* was synthesized and added to the hybridization buffer. This probe would have a much higher affinity to the 16S rRNA of *P. aeruginosa* than would Eco16S07C-SBP and was expected to block the mismatch hybridization by outcompeting Eco16S07C-SBP. An SBP-labeled version of this PNA blocker probe targeted only *P. aeruginosa* (Fig. 3). By using a 100-fold excess of the PNA blocker probe, the mismatch hybridization was eliminated without decreasing the specific hybridization of Eco16S07C-SBP to *E. coli* (Fig. 3).

Sensitivity and specificity of *E. coli* PNA CISH assay. Sensitivity of *E. coli* PNA CISH with the probe was evaluated against eight *E. coli* strains. All isolates were positively identified as *E. coli*, although strain HF174 yielded a relatively weak signal (Table 1). As smaller colonies were obtained for this strain than for the other *E. coli* strains following overnight incubation, this weak signal was considered to be due to a lower growth rate resulting in smaller microcolonies after 5 h of growth rather than to variability in the target 16S rRNA sequence.

Subsequently, the specificity of *E. coli* PNA CISH was evaluated using strains of closely related organisms, as well as environmentally and clinically relevant microorganisms. The Eco16S07C-SBP probe did not react with other bacterial species tested, except with *Shigella* strains in accordance with sequence data (Table 1). *P. aeruginosa* was not detected by the probe, although data described above showed a mismatch hybridization of Eco16S07C-SBP to *P. aeruginosa* rRNA. For all bacteria, visible colonies were obtained following overnight incubation.

By increasing the level of *P. aeruginosa* to more than 10^3 CFU membrane, a hybridization signal was obtained (data not shown). However, this signal from up to 10^5 CFU membrane was blocked by inclusion of a 100-fold excess of the PNA blocker probe alongside Eco16S07C-SBP without affecting the specific signal obtained with *E. coli* (data not shown).

Detection of culturable *E. coli* by PNA CISH. Approximately 70 CFU of *E. coli* were filtered onto membrane filters that were not incubated or were incubated on TSA at various temperatures to promote or inhibit growth (Table 2). *E. coli* was detected by PNA CISH and standard culture methods following 5-h and overnight incubation, respectively, on TSA at 35°C. *E. coli* was not detected by either method when incubated at 4°C but was still viable as determined by the formation of colonies following subsequent overnight incubation at 35°C after the initial 5-h incubation at 4°C. Moreover, *E. coli* was not detected by PNA CISH following filtration without incubation.

Correlation with colony counts. Correlation between the PNA CISH method and colony counts was established by evaluating dilutions of *E. coli* filtered directly or spiked into 100 ml of filter-sterilized municipal water or 100 ml of municipal water (Fig. 4). The control assays of 100 ml of filter-sterilized municipal water and 100 ml of municipal water were also performed. Representative X-ray images are shown in Fig. 5. Colony counts of *E. coli* spiked into 100 ml of municipal water were complicated by other colony-forming microorganisms present in the tap water samples. Colonies that were morphologically different from *E. coli* were not counted. The slope of the linear regression lines indicates 92 to 95% correlation of PNA CISH with colony counts.

The use of R2A and LB agar as alternative growth media was investigated prior to the PNA CISH. Samples of *E. coli* were filtered and incubated on R2A, TSA, and LB agar at 35°C for 5 h. Not only did the number of CFU determined by PNA CISH and colony counts correlate well for any given medium, but all media were also found to be comparable to each other given the aforementioned growth conditions (Table 3).

TABLE 2. Enumeration of *E. coli* cells by PNA CISH and culture following various growth conditions on TSA[a]

Culturability	Growth condition	PNA CISH (no. of spots of light)	Culture (no. of colonies)
Promoting	5 h at 35°C	69 ± 9.0	
Promoting	O/N[b] at 35°C		72 ± 3.0
Inhibitory	5 h at 4°C	0.33 ± 0.58	
Inhibitory	O/N at 4°C		0 ± 0
Promoting	5 h at 4°C + O/N at 35°C		70 ± 7.5
None	No growth	0.33 ± 0.58	

[a] Each value is the mean with 1 standard deviation based on three determinations.
[b] O/N, overnight.

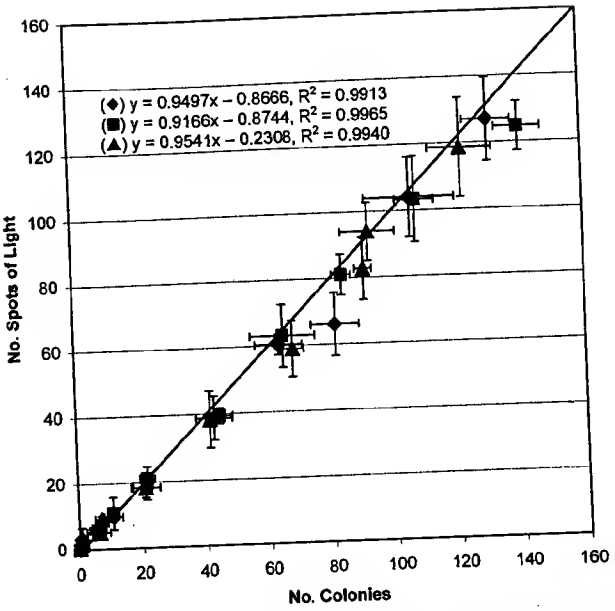

FIG. 4. Correlation of CFU of *E. coli* as determined by PNA CISH and culture. ◆, *E. coli*; ■, *E. coli* spiked into 100 ml of filter-sterilized municipal water; ▲, *E. coli* spiked into 100 ml of municipal water. Each data point is the mean of three determinations with error bars of 1 standard deviation. The diagonal line represents 100% correlation.

TABLE 3. Enumeration of *E. coli* cells by PNA CISH and colony counts from membrane filters incubated on TSA, R2A, and LB agar[a]

Growth medium	PNA CISH (no. of spots of light)	Culture (No. of colonies)
TSA	47 ± 5.6	59 ± 5.6
R2A	49 ± 5.1	51 ± 7.9
LB agar	55 ± 5.7	49 ± 4.0

[a] Each value is the mean with 1 standard deviation based on three determinations.

DISCUSSION

Rapid methods for microbiological analysis of drinking water must not only provide the same type of information as that from current methods, such as detection, identification, and enumeration, but also do so faster. PNA CISH rapidly and simultaneously detects, identifies, and enumerates *E. coli* cells within one working day and is to our knowledge the only method that has these attributes. PNA CISH combines the advantages of membrane filtration for capturing microorganisms from large sample volumes and a growth step for distinguishing between culturable and nonculturable microorganisms with the sensitivity and specificity provided by the PNA CISH technology.

The PNA CISH method relies on whole-cell in situ hybridization of the SBP-labeled PNA probe to individual microcolonies, the growth of which is terminated well before they are visible by eye. Results are provided as spots of light whose number and location relate to colonies of *E. coli* traditionally recovered and enumerated by sustained growth. Regardless of whether a pure *E. coli* culture or spiked municipal water was being analyzed, the mean and variability of the spots of light generated by PNA CISH correlated well with those of *E. coli* colonies formed by standard culture methods. This good correlation is ascribed to the growth of microcolonies prior to performance of PNA CISH such that only viable, growing cells are detected. Comparable data were also obtained using different growth media. This suggests that PNA CISH can replace traditional culture methods without necessitating a change in growth media. PNA CISH is therefore easily compared with current methods to provide a more rapid result. Although the data suggest that 5 h of incubation prior to PNA CISH is sufficient for various growth media such as R2A, TSA, and LB agar, culturing conditions such as incubation temperature and time should always be optimized for a particular microorganism, sample type, and growth medium.

Identification of microorganisms based upon 16S rRNA sequences is today a widely accepted method and is rapidly replacing conventional identification that relies on morphological and phenotypic characteristics. Using this approach, strains with atypical phenotypes, such as MUG-negative *E. coli* strains, can be detected (2,). The excellent specificity of PNA probes for definitive identification of *E. coli* as demonstrated in this study is consistent with several other studies using PNA probes showing specificities of 100% (18, 19–21).

However, as with most other identification methods, there are some limitations that must be considered. For example, a 100% *E. coli*-specific 16S rRNA target sequence does not exist, as the 16S rRNA sequences of *E. coli* and *Shigella* spp. are almost identical. A probe designed to detect *E. coli*, but not *Shigella* spp., based upon 16S rRNA sequence differences that

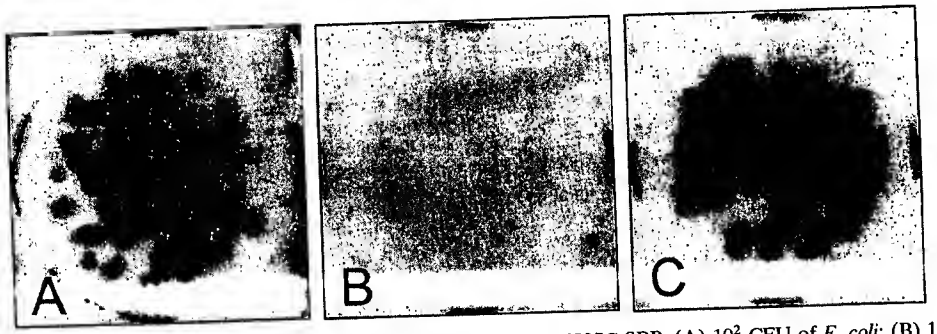

FIG. 5. Representative images of results obtained with PNA CISH using Eco16S07C-SBP. (A) 10^2 CFU of *E. coli*; (B) 100 ml of municipal water; (C) 10^2 CFU of *E. coli* spiked into 100 ml of municipal water.

do exist would then detect several other bacteria, in particular bacteria within the *Enterobacteriaceae* family. This lack of 100% species-specific rRNA target sequences of *E. coli* is well known (22). Although *Shigella* spp. will lead to false-positive test results following *E. coli* PNA CISH, they are serious human pathogens that should not be present in drinking water.

Another limitation is mismatch hybridization as seen between Eco16S07C-SBP and the 16S rRNA of *P. aeruginosa*. The use of a PNA blocker probe was able to completely eliminate this mismatch hybridization. For the analysis of municipal water, the use of PNA blocker probe may not be required, as this mismatch hybridization was observed only in the dot hybridization assay and the PNA CISH assay when more than 10^3 CFU of *P. aeruginosa* was filtered. A similar application of nonlabeled PNA blocker probes has previously been used to increase the signal-to-noise ratio by decreasing mismatch hybridization without affecting specific hybridization (9).

The high degree of variability of the nonspecific background spots obtained with the two different PNA probes suggests that the sequence-base composition of the PNA probe is responsible for this. Further studies are ongoing to explore this phenomenon in more detail. However, as with other types of analyte-specific reagents such as antibodies and DNA probes, the nonspecific binding to a given sample matrix must be evaluated before the specificity and sensitivity of an assay can be determined.

The speed of the PNA CISH method allows for faster responses in the event of bacterial contamination of water, thereby reducing the negative consequences of contamination regardless of where in the process the water is being tested. This is particularly important for final testing of water prior to release to the public. Moreover, unnecessary concerns due to presumptive identification of nonpathogenic-nonfecal coliforms and subsequent time required for verification are eliminated (5).

X-ray film was used for capturing the light signal from each microcolony as this allowed multiple membranes to be exposed simultaneously. However, laboratories performing routine microbiological analysis of municipal water may not be equipped with a film developer and darkroom, and other detection systems should be considered. The MicroStar System (Millipore) and a bench top camera box with high-speed instant film have both been applied to the PNA CISH technology and constitute excellent alternatives for routine applications in different laboratory settings (20).

In conclusion, the PNA CISH method for rapid and simultaneous detection, enumeration, and identification of *E. coli* cells in municipal water enables the use of rapid methods for microbial analysis of municipal water. Unlike other rapid microbiological methods, it provides the same information (detection, identification, and enumeration) as do current methods without compromising that information for speed. This feature should allow *E. coli* PNA CISH to be validated directly against current standard methods according to existing acceptance criteria.

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